

NKX6.1 induced pluripotent stem cell reporter lines for isolation and analysis of functionally relevant neuronal and pancreas populations

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ABSTRACT

Recent studies have reported significant advances in the differentiation of human pluripotent stem cells to clinically relevant cell types such as the insulin producing beta-like cells and motor neurons. However, many of the current differentiation protocols lead to heterogeneous cell cultures containing cell types other than the targeted cell fate. Genetically modified human pluripotent stem cells reporting the expression of specific genes are of great value for differentiation protocol optimization and for the purification of relevant cell populations from heterogeneous cell cultures. Here we present the generation of human induced pluripotent stem cell (iPSC) lines with a GFP reporter inserted in the endogenous NKX6.1 locus. Characterization of the reporter lines demonstrated faithful GFP labelling of NKX6.1 expression during pancreas and motor neuron differentiation. Cell sorting and gene expression profiling by RNA sequencing revealed that NKX6.1-positive cells from pancreatic differentiations closely resemble human beta cells. Furthermore, functional characterization of the isolated cells demonstrated that glucose-stimulated insulin secretion is mainly confined to the NKX6.1-positive cells. We expect that the NKX6.1-GFP iPSC lines and the results presented here will contribute to the further refinement of differentiation protocols and characterization of hPSC-derived beta cells and motor neurons for disease modelling and cell replacement therapies.

1. Introduction

Human embryonic and induced pluripotent stem cells (hESC and hiPSC, together hPSC) provide a novel avenue for the generation of disease-relevant cell types such as pancreatic beta cells and motor

neurons useful for disease-modelling, drug screening and cell replacement therapy (Cohen and Melton, 2011; McNeish et al., 2015). In diabetes, the insulin-producing beta cells of the pancreas are either of insufficient numbers to meet the required metabolic demand, dysfunctional or selectively destroyed (Christofferson et al., 2016; Yang

Abbreviations: EBiSC, European Bank for Induced Pluripotent Stem Cells; FACS, Fluorescence activated cell sorting; GFP, Green fluorescence protein; GSIS, Glucose stimulated insulin secretion; HBSS, Hank's balanced salt solution; HDR, Homology-directed repair; hESC, Human embryonic stem cells; hiPSC, Human induced pluripotent stem cells; hPSC, Human pluripotent stem cells; IBMX, 3-isobutyl-1-methylxanthine; IMI, Innovative Medicine Initiative; KCl, Potassium chloride; KRBH, Krebs-Ringer bicarbonate hepes buffer; NEO, Neomycin-resistance gene; NKX6.1, NK6 homeobox 1; PBS, Phosphate-buffered saline; PDX1, Pancreatic and duodenal homeobox 1

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and Chan, 2016). Unfortunately, human beta cells can only be isolated in limited numbers from cadaveric donor pancreas, and are of varying quality. Thus, research in development, progression and treatment of diabetes could greatly benefit from novel sources of human beta cells. A similar issue persists for cellular models of neurodegenerative disorders, like amyotrophic lateral sclerosis and spinal muscular atrophy, where the motor neurons affected by the disease can only be derived from *post mortem* patient sources.

Recent advances in the development of differentiation protocols for hPSC now allow for the derivation of beta-like cells and motor neurons that share many of the functional and molecular characteristics that define their *in vivo* counterpart (Rezania et al., 2014; Pagliuca et al., 2014; Russ et al., 2015; Wichterle et al., 2002; Di Giorgio et al., 2008; Amoroso et al., 2013). Despite these advances, it is becoming clear that the hPSC-derived beta-like cells have shortcomings compared to human beta cells (Kushner et al., 2014; Johnson, 2016) and the efficiency of the differentiation protocols can be variable depending on the hPSC lines used (Osafune et al., 2008; Nostro et al., 2015; Nishizawa et al., 2016; Boulting et al., 2011). This indicates that further development and refinement of the differentiation protocols for deriving relevant cell types from hPSC are needed. Genetically modified hPSCs designed to express a fluorescent reporter gene under the control of cell-type specific promoters facilitate identification and isolation of relevant cell types in otherwise heterogeneous cultures (Giudice and Trounson, 2008). Thus, generation of hPSC lines reporting the expression of genes important for beta cell and motor neuron development has the potential to greatly aid future efforts aimed at improving and characterizing the differentiation of hPSC towards these cell types, as well as allow for their purification.

NK6 homeobox 1 (*NKX6.1*) is expressed broadly in the pancreatic epithelium during development and subsequently becomes restricted to the beta cells, where it is required for the expression of genes critical for beta cell function and identity (Pedersen et al., 2005, 2006; Jorgensen et al., 2007; Jennings et al., 2013; Taylor et al., 2013). *NKX6.1* is also expressed in other non-pancreas tissues during embryonic development and has been shown to be involved in the specification of motor neurons in the developing nervous system (Sander et al., 2000), as supported by reports of *NKX6.1* expression during directed differentiation of hESC into motor neurons (Salehi et al., 2009; Gonzalez-Garza et al., 2013).

Several lines of evidence suggest that *NKX6.1* also plays a critical role in the specification of pancreatic endoderm, and subsequently of beta cells during the differentiation of hPSC towards the pancreatic lineage. Many studies have demonstrated that hESC-derived pancreatic endoderm expressing *NKX6.1* and pancreatic and duodenal homeobox 1 (*PDX1*) had the competence to differentiate into functional beta cells and other pancreas lineages following transplantation into immunocompromised mice (Russ et al., 2015; Nostro et al., 2015; Kroon et al., 2008; Rezania et al., 2012; Kelly et al., 2011; Rezania et al., 2013). Furthermore, the recently described differentiation protocols for the derivation of beta-like cells were developed by focusing on promoting endocrine differentiation from *NKX6.1*-expressing pancreatic endoderm (Rezania et al., 2014; Pagliuca et al., 2014; Russ et al., 2015). Thus, future studies aimed at optimizing the differentiation protocols for deriving fully functional beta cells from hPSC are likely to revolve around promoting and maintaining the expression of the transcription factor *NKX6.1*.

Here we describe the generation and validation of *NKX6.1*-green fluorescence protein (GFP) reporter lines in a hiPSC line derived from a healthy donor. The expression of GFP protein is driven by the endogenous *NKX6.1* promoter, and the reporter construct does not interfere with endogenous *NKX6.1* expression levels, thus eliminating the risk of a haploinsufficiency phenotype. Furthermore, we demonstrate that these reporter lines are useful for monitoring, isolation and characterization of hiPSC-derived *NKX6.1*-expressing cell types, including beta-like cells and motor neuron lineage. These reporter lines will be

made available to the scientific community through the Innovative Medicine Initiative (IMI) sponsored, private-public partnership project StemBANCC and could provide a valuable resource for deriving and characterizing pancreas and motor neuron progenitors as well as functional pancreatic beta cells for disease modelling and drug discovery studies.

2. Material and methods

2.1. Gene editing of iPSC lines

The Wild type SBAD03-01 and SBAD03-04 iPSC (both lines referred to as SB AD3 (WT) in manuscript) were obtained through the IMI/EU sponsored StemBANCC consortium via the Human Biomaterials Resource Centre, University of Birmingham (<http://www.birmingham.ac.uk/facilities/hbrc>). SBAD03-01 iPSC line was used for generation of *NKX6.1*-GFP reporter lines. SBAD03-01 cells were harvested at passage P12 using TrypLE and 300,000 cells/well of a 12-well plate were transfected with targeting plasmid vector and CRISPR/Cas9 plasmid vector DNA using Lipofectamine LTX (ThermoFisher). Transfected cells were passaged once confluence and selection with G418 (0.1 mg/ml) was performed after 24 h. When G418-resistant colonies expanded, single cell cloning was performed through serial dilution in 384 well plates to identify pure clone. Correctly targeted clones were identified by junction PCR and ddPCR and confirmed by Sanger sequencing analysis as described in the supplemental material and methods.

2.2. Directed differentiation of iPSC lines

Cells were differentiated to the pancreatic lineage according to protocol developed by Rezania et al. (Rezania et al., 2014). Briefly, iPSCs were seeded as a single cell solution onto Corning CellBind surfaces (Corning) coated with growth factor reduced matrigel (Corning #356230) in TeSR1-E8 medium with 5uM Rock Inhibitor (Tiger, Sigma-Aldrich #Y0503). Cells were seeded at $0.3\text{--}0.35 \times 10^6$ cells pr. cm^2 and differentiation was started 24 h post seeding of cells. Cells were rinsed once in PBS (with calcium and magnesium, PBS+/+) immediately before starting the differentiation. Cells were differentiated in the culture plates for all stages of the differentiation and medium was replenished every 24 h. Details on compounds and medium formulation used for the differentiation to the pancreatic lineage as well as details for differentiation to the motor neuron lineage are listed in the Supplemental material and methods and key resource table.

2.3. Immunofluorescence imaging and flow cytometry

Immunofluorescence and flow cytometry analysis of cells was carried out essentially as described in (Honore et al., 2016) and described in detail in the Supplemental material and methods and key resource table.

2.4. Fluorescence-activated cell sorting (FACS)

For cell sorting, pancreas stage 7 cells were dissociated to a single cell solution using TrypLE select and washed once. Cells were resuspended in differentiation medium (MCDB131-3, without factors) containing 0.1 $\mu\text{g}/\text{ml}$ DAPI solution (BD Biosciences, #564907). Cells were sorted on a BD FACSAria Fusion cell sorter (BD Biosciences) using a 100uM nozzle.

2.5. Dynamic glucose stimulated insulin secretion assay

Dynamic glucose stimulated C-peptide, human insulin and glucagon release was investigated using the Biorep Perfusion system 4.2 according to manufacturer's guidelines. Clusters and human islets were loaded into columns; to ensure proper loading, clusters were enclosed

by Bio-Gel P-4 beads (Biorad). After 60 min. of acclimatization in KRBH buffer (Ampliqon; 2 mM Glutamax + 0.2% (w/v) HSA) supplemented with 2 mM glucose (Sigma) at flow 50 μ l/min (kept constant during the experiment), clusters were exposed to different glucose concentrations, IBMX(500 μ M, Sigma) + forskolin (10 μ M, Tocris) stimulation in the presence of 20 mM glucose was used to increase intra-cellular cAMP concentration. Secretory output was collected at defined intervals and afterwards the islets were collected, washed in HBSS (Gibco) and lysed (tissue extraction reagent I Invitrogen) + tissue lyzer (Qiagen). Procedure for determination of C-Peptide, human insulin and glucagon concentrations are described in Supplemental material and methods.

3. Results

3.1. Generation of NKX6.1-GFP reporter iPSC lines

We generated NKX6.1-GFP knockin reporter lines by targeting the NKX6.1 locus using neomycin drug selection (Fig. S1A). The StemBANCC iPSC line SBAD03-01 selected for targeting was derived from a healthy donor, showed a stable karyotype and efficiently differentiated towards the pancreatic lineage (data not shown). SBAD03-01 was co-transfected with two plasmids: one expressing Cas9 and a crRNA/tracrRNA duplex targeting NKX6.1, and one containing the fluorescent reporter and a drug-resistance cassette as the HDR template (Fig. 1A and Fig. S1B). We used a donor plasmid, T2A-GFP-PGK-Neo, in which the last NKX6.1 coding codon is fused in frame with a T2A sequence followed by GFP (T2A-eGFP) and a loxP-flanked (floxed) neomycin-resistance gene expressed from the constitutive PGK promoter (PGK-Neo) (Fig. 1A and Fig. S1B). This strategy minimizes any potential impact on the endogenous protein and is applicable to targeting both silent and expressed genes. Junction PCR using primers spanning endogenous and reporter construct DNA as well as ddPCR analysis identified 9 correctly targeted clones without random transgene integration from a total of 120 neomycin-resistant clones screened (Fig. 1B and C). The targeting efficiency (7%) was comparable to the efficiencies observed with TALENs and ZFNs using similar targeting strategies (Hockemeyer et al., 2009, 2011). NKX6.1-GFP (+Neo) clones 1a-10 (with two copy insertion) and 1a-21 (with single copy insertion) were proceeded for neomycin removal. After Cre-mediated excision of the PGK-Neo cassette, almost all resulting NKX6.1-GFP (-Neo) clones showed absence of *neo* in ddPCR and Sanger sequencing analysis (Fig. 1C and D). NKX6.1-GFP (-Neo) clones 1a-10-3 (two copy insertion) and 1a-21-3 (single copy insertion) were chosen for further characterization. Immunofluorescence microscopy showed expression of OCT4, NANOG, Tra-1-60 and Tra-1-81 and karyotype analysis revealed a normal karyotype in both NKX6.1-GFP (-Neo) clones (Fig. 1E).

3.2. NKX6.1-GFP iPSC lines label NKX6.1 expressing cells during pancreatic differentiation

In order to confirm that the NKX6.1-GFP construct would faithfully report NKX6.1 expression we applied an established protocol for differentiating the NKX6.1-GFP iPSC lines towards the pancreatic lineage (Rezania et al., 2014). Wild type and the two NKX6.1-GFP iPSC clones (clone 1a-10-3 and 1a-21-3) were differentiated towards pancreatic endoderm where NKX6.1 is known to be expressed (Rezania et al., 2014). We observed a GFP signal in live NKX6.1-GFP iPSC-derived pancreatic endoderm both by fluorescence microscopy and flow cytometry, whereas no signal was detected in the wild type iPSC line differentiated to the same stage (Fig. 2A and B). Importantly, a clear overlap between the GFP signal and endogenous expression of NKX6.1 was observed by immunofluorescence microscopy (Fig. 2C). NKX6.1-positive cells also expressed PDX1 and SOX9 (Fig. S2A), two transcription factors known to be expressed in pancreatic endoderm, demonstrating differentiation to the pancreatic lineage. When assessing the percentage of NKX6.1 and GFP positive cells by flow cytometry, we

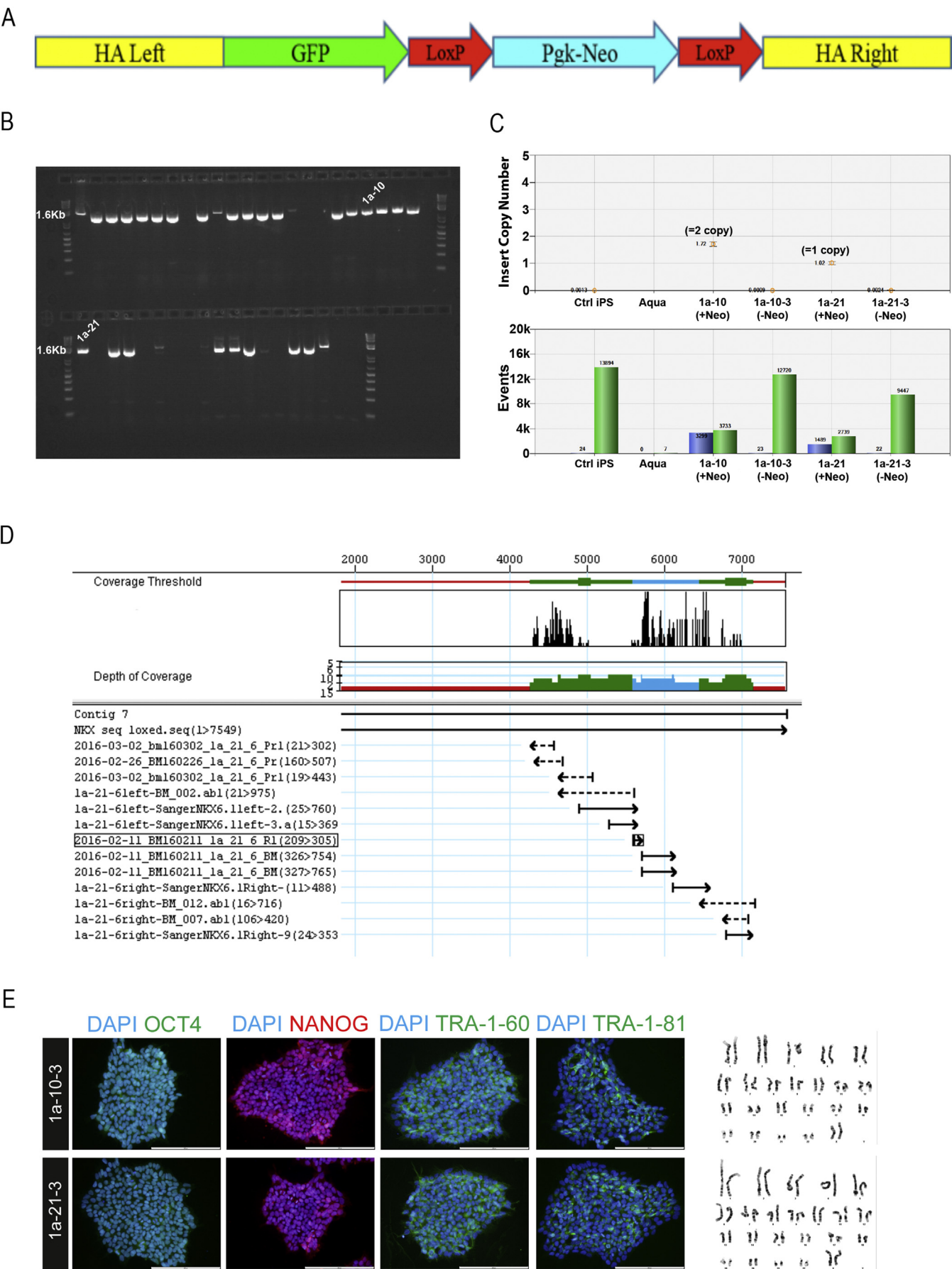
observed co-expression of GFP and NKX6.1 in 83–85% of the cells expressing NKX6.1 (Fig. 2D and E). Together, these results demonstrate that the reporter lines faithfully label NKX6.1 expression during differentiation towards the pancreatic lineage.

3.3. Differentiation of NKX6.1-GFP iPSC lines to insulin producing Beta-like cells

To test the ability of the NKX6.1-GFP iPSC lines to generate insulin-producing beta-like cells we employed a seven-stage differentiation protocol that allows for the derivation of beta-like cells from hPSC (Rezania et al., 2014). At stage 7 of the protocol we observed robust induction of C-peptide expression in both wild type and NKX6.1-GFP iPSC lines (Fig. 3A). C-peptide was used as a marker for detecting endogenous insulin production (Rajagopal et al., 2003; Hansson et al., 2004). Approximately 25–30% of the cells expressed C-peptide and notably between 60 and 70% of the C-peptide positive cells were GFP positive in the NKX6.1-GFP lines, indicating co-expression of C-peptide and NKX6.1 (Fig. 3A and B). Co-expression of C-peptide and GFP was also confirmed by immunofluorescence microscopy (Fig. 3C). The iPSC-derived beta-like cells could secrete insulin in response to elevated glucose concentrations as well as arginine or KCl (Fig. S2B and S2C). Importantly, > 85% of the NKX6.1 positive cells co-expressed GFP, confirming the faithfulness of the reporter lines in labelling NKX6.1 expression at this stage of the differentiation protocol (Fig. 3D and Fig. S2D). The relative levels of endogenous NKX6.1 protein expression were assessed by flow cytometry and we observed no differences in NKX6.1 expression levels when comparing wild type and the NKX6.1-GFP iPSC lines (Fig. 3E and F). These results demonstrate that the NKX6.1-GFP iPSC lines are capable of differentiating into insulin producing beta-like cells at comparable efficiency to the wild type iPSC lines from which they were derived.

3.4. Gene expression profiling demonstrates transcriptional similarities between NKX6.1 positive cells and human Beta cells

GFP positive and negative cell populations were enriched to high purity from stage 7 of the differentiation protocol using fluorescence activated cell sorting (FACS) (Fig. 4A and B). Real time quantitative qPCR confirmed an accompanying enrichment or depletion of NKX6.1 transcript in the GFP positive and negative cells, respectively (Fig. S3A). In order to further characterize the two populations and compare with human islet populations we performed whole transcriptome profiling using RNA sequencing. Briefly, we collected the RNA from a total of five differentiations of the two different clones of the NKX6.1-GFP cell line differentiated to stage 7, and FACS-sorted into NKX6.1-GFP positive and NKX6.1-GFP negative populations. We prepared SmartSeq2 paired-end RNA-seq libraries for a total of 15 samples (5 pre-sorted cell populations, 5 NKX6.1-GFP positive, 5 NKX6.1-GFP negative), and sequenced on Illumina HiSeq4000 to a mean depth of 36.9 million (\pm 0.9 million) 75 bp read pairs per sample. We observed a consistent transcriptomic profile of the GFP positive cells from five independent differentiations. In multidimensional scaling plot of the whole transcriptome data those samples clustered together, and were separate from the GFP negative and unsorted cell populations (Fig. 4C). Several key beta cell markers and transcription factors such as *PDX1*, *GLP1R*, *IAPP*, *PCSK1* and *SLC30A8* were enriched in the GFP positive cells and expressed at comparable levels to human islets (Fig. 4D). However, we also noted few known beta cell markers with lower expression in the iPSC-derived populations compared to human islets (e.g. *MAFA* and *G6PC2*). *ARX*, a transcription factor recently shown to be expressed in both human alpha and pancreatic polypeptide (PP) cells by single cell RNA-seq (Li et al., 2016; Segerstolpe et al., 2016; Baron et al., 2016) as well as *GCG*, *PPY* and *GHRL* were enriched in the GFP negative population (Fig. 4D), suggesting that the GFP negative population contains the majority of cells expressing markers of alpha, PP and epsilon cells



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Fig. 1. CRISPR/Cas9-Mediated Targeting of the *Nkx6.1* Locus through Drug Selection. A) Schematics of the targeting strategy. In the presence of the donor plasmid, HDR results in the replacement of the *Nkx6.1* stop codon with T2A-GFP-LoxP-PGK-Neo-LoxP cassette. In the targeting schematics here, coloured boxes are various segments of the insert, HA Left and HA Right indicate left and right homology arms. B) The PCR primers (F + GFP-R) used for genotyping for identifying correctly targeted clones. Two of the correctly targeted clones (clone 1a-10 and clone 1a-21) were selected for transfection with Cre recombinase to remove Neomycin cassette. C) ddPCR analysis indicating insert copy number. Internal neomycin probe was used for analyzing the clones, showed zero insert copy number for control iPSC, two copies for 1a-10 and one copy for 1a-21 clone. After Cre mediated excision clone 1a-10-3 of 1a-10 and clone 1a-21-3 of 1a-21 showed complete removal of neomycin cassette. Blue bar represents total events number for neo probe and the green bar represents total number of events for reference gene *AP3B1*. Aqua sample had no template DNA. D) Sanger sequencing through overlapping amplicons spanning outer junctions of both homology arms and the entire insert showing localization and integrity of the insert. E) Immunofluorescence images and karyotype analysis of NKX6.1-GFP clones 1a-10-3 and 1a-21-3. DAPI is shown in blue. Scale bar = 200 μ M. See also Fig. S1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 4D). The mutual exclusive expression of *GFP* and *GCG* was also confirmed on protein level using both flow cytometry and immunofluorescence imaging (Fig. S3B and S3C). Interestingly, we observed the expression of the delta cells marker somatostatin (*SST*) in both the GFP positive and negative populations, with the highest expression observed in the GFP positive population (Fig. 4D). We confirmed expression of somatostatin in both the GFP positive and negative fractions both by flow cytometry and immunofluorescence microscopy analysis (Fig. S3D and S3E).

In order to test whether enriching for NKX6.1-positive cells from the differentiation cultures would yield a cell population more closely resembling human beta cells on the transcriptional level, we compared our sorted populations to a published data set of mature human islet cells FACS-sorted into three distinct populations: beta cells, non-beta cells and whole islets (Nica et al., 2013) (Fig. 4E). We observed that the iPSC-derived NKX6.1-positive cells very closely resembled the human beta cells, whereas the NKX6.1-negative and unsorted populations were more similar to the non-beta cell and whole islet populations, respectively (Fig. 4E). We then compared the transcriptomic profiles of the GFP positive and GFP negative population specifically, and discovered 6000 genes significantly differentially expressed at 5% FDR (Table S1). We noted significant differential expression of *INS*, *PPY* and *GCG* endocrine hormones, and observed the same distribution in the mature human islet cells populations (Fig. S4). Overall, the magnitude of differences observed between the GFP-positive and GFP-negative cell populations was similar to those between the beta and non-beta mature human islet cells, as evidenced by significant correlation of the log2 fold changes from the respective differential expression analyses ($r = 0.32$, $p < 2.2e-16$) (Fig. 4F). These results demonstrate that the NKX6.1-GFP reporting faithfully labels the cellular populations present in hiPSC cell lines differentiated *in vitro*, which closely resemble the FACS-sorted beta and non-beta mature human islet cells, and facilitates exploration of the differences between those populations.

3.5. Glucose-responsive cells are present in the NKX6.1-positive population

Since our transcriptional profiling suggested that the NKX6.1-positive population were more closely related to human beta cells compared to the NKX6.1-negative population, we also sought to determine whether this was reflected by glucose stimulated insulin secretion. To this end, we FACS-purified GFP positive and negative cells from stage 7 cultures. Sorted, single cell suspensions along with unsorted cells were aggregated overnight to clusters of approximately 50–150 μ M in diameter (Fig. 5A). We confirmed the purity of GFP positive and negative cells compared to the unsorted cells by fluorescence microscopy following the overnight aggregation (Fig. 5A). Analysis of total protein content of C-Peptide and glucagon in the sorted cell populations following aggregation and perfusion confirmed that glucagon was primarily expressed in the GFP negative population (Fig. 5B) as expected based on our results above (Fig. 4D, Figs. S3B, S3C, S4). C-peptide was detected in both the GFP negative and positive population, with a higher concentration in the latter (Fig. 5B).

To assess the functionality, we subjected the sorted and aggregated clusters to a dynamic glucose stimulated insulin secretion (dynamic GSIS) challenge by perfusion. Across three independent experiments,

we observed a basal C-peptide secretion from both the unsorted and the GFP positive populations in 2 mM glucose concentration, while a significantly lower basal secretion was detected from the GFP negative population. Furthermore, when the cells transitioned from 2 mM to 20 mM glucose concentration we noticed an approximately 2-fold increase in the C-peptide secretion from the GFP positive and unsorted populations, while no increase in secretion was observed from the GFP negative population (Fig. 5C). We did not observe any significant differences in the levels of C-peptide secreted from the GFP positive population compared to the unsorted (Fig. 5C). However, the consistent 2-fold increase in C-peptide secreted when transitioning from 2 mM to 20 mM glucose suggested that cells within both of these populations secreted insulin in response to elevated glucose concentrations. Of note, the glucose stimulated C-peptide secretion from the NKX6.1-GFP positive and unsorted populations was significantly lower compared to insulin secretion from human islets exposed to the same glucose concentration and (Fig. S5A). When the cells were exposed to compounds increasing intracellular cAMP content in the presence of 20 mM glucose we observed a substantial increase in C-peptide secretion from both the unsorted and GFP positive populations, but also from the GFP negative populations albeit at much lower levels (Fig. 5C). When assessing glucagon, we observed secretion from both the unsorted and the GFP negative population, while we could not detect any glucagon secretion from the GFP positive population (Fig. 5D). We did observe secretion of comparable levels of glucagon from human islets but in contrast to the hPSC-derived populations the secretion of glucagon from human islets appeared to be suppressed by high glucose concentrations (Fig. S5B). Increasing cAMP in the presence of high glucose led to a significant increase in glucagon secretion from both the unsorted and GFP negative populations, with only very small amounts of glucagon secretion detected from the GFP positive population (Fig. 5D). Together, these results demonstrate the presence of cells secreting insulin in response to elevated glucose concentrations within the NKX6.1 population.

3.6. Differentiation of NKX6.1-GFP iPSC lines to motor neuron lineage

In addition to its role in pancreatic development, NKX6.1 is also shown to be a key regulator of motor neuron specification in the developing brain (Sander et al., 2000; Vallstedt et al., 2001). Therefore, to examine if the NKX6.1-GFP construct would also report NKX6.1 expression in motor neuron precursors we differentiated NKX6.1-GFP reporter iPSC lines towards the motor neuron lineage using a previously published differentiation protocol (Wichterle et al., 2002; Di Giorgio et al., 2008; Amoroso et al., 2013; Boulting et al., 2011). At day 15 of the 4 weeks long differentiation protocol, we observed maximum GFP signal by fluorescence microscopy in the live NKX6.1-GFP iPSC-derived embryoid bodies, whereas no signal was detected in the wild type iPSC lines differentiated to the same stage (Fig. 6A). The embryoid bodies were dissociated into single cells at day 15 for further characterization of various motor neuron lineage specific markers. As expected, we observed an overlap between the GFP signal and endogenous expression of NKX6.1 by immunofluorescence microscopy in each of clones 1a-10-3 and 1a-21-3 (Fig. 6B). A fraction of GFP and *Nkx6.1*-positive cells also expressed *ISL1* (Fig. 6B), a transcription factor known to be expressed in early motor neurons, hereby demonstrating successful

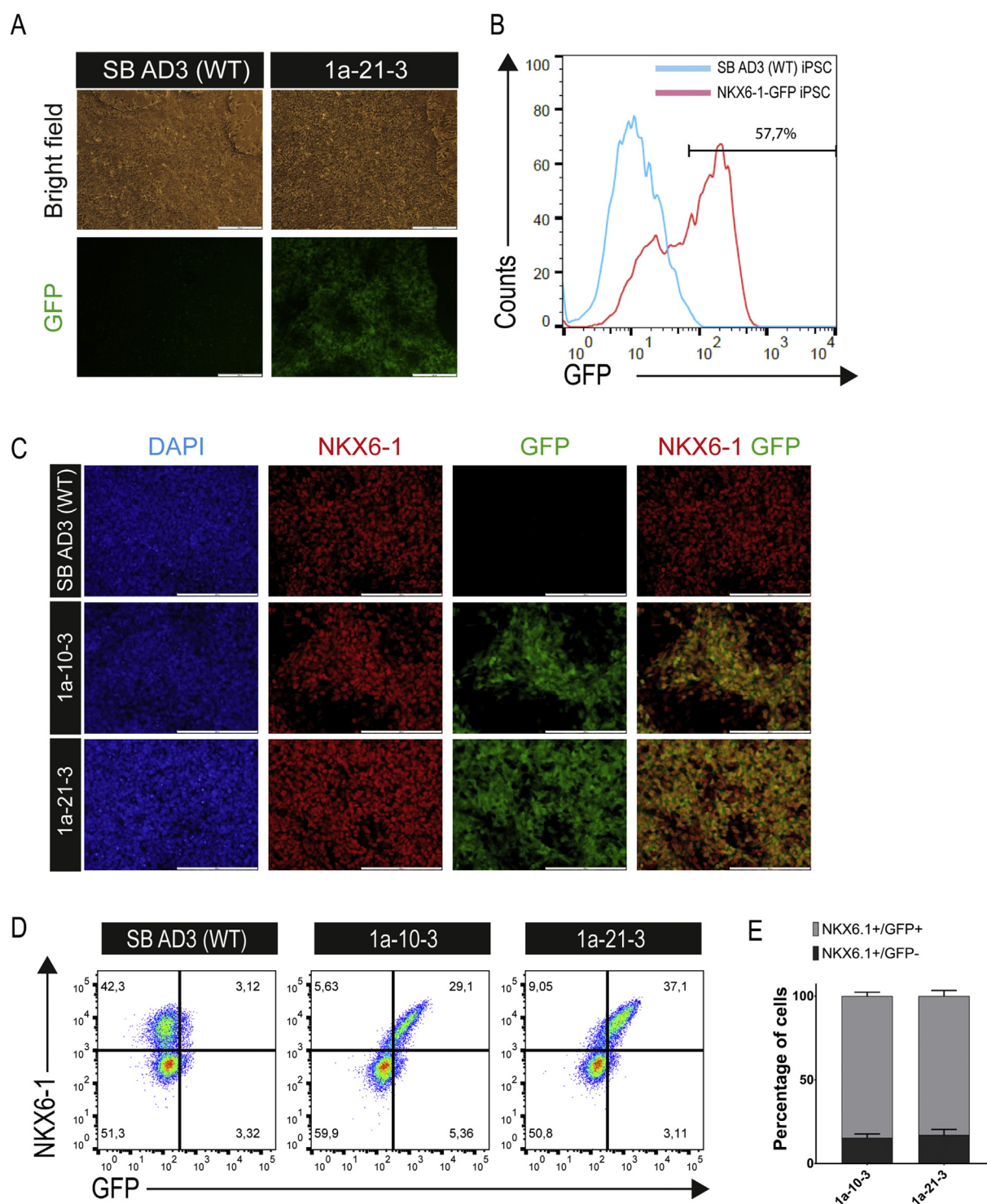


Fig. 2. Characterization of the NKX6.1-GFP lines in pancreatic endoderm differentiation. Wild type (SB AD3.1 and SB AD3.4) and NKX6.1-GFP iPSC lines (1a-10-3 and 1a-21-3) were differentiated to pancreatic endoderm and analysed for GFP expression and pancreatic endoderm markers by immunofluorescence microscopy and flow cytometry. **A)** Representative bright field and fluorescence microscopy images of live cultures of SB AD3.1 and 1a-21-3 pancreatic endoderm. Scalebar = 200uM. **B)** Representative histogram of live wild type (Blue) and NKX6.1-GFP iPSC lines (Red) analysed for GFP expression by flow cytometry. X-axis shows GFP fluorescence intensity, Y-axis shows number of events (counts). Gating was set based on < 1% of the signal from the wild type iPSC line. Number shows percentage of GFP-positive cells of the NKX6.1-GFP iPSC line. **C)** Wild-type and NKX6.1-GFP lines stained for NKX6.1 and GFP protein. Nuclei were revealed with DAPI. **D)** Flow cytometry dot-plots of NKX6.1 protein (Y-axis) and endogenous GFP signal (X-axis). Numbers indicate percentage of cells in each quadrant. Representative dot-plots of 3 (1a-10-3) and 5 (both 1a-21-3 and WT) experiments. **E)** Percentage of NKX6.1 positive cells positive (light grey) or negative (dark grey) for GFP expression as assessed in D). Graph shows mean \pm SD, $n = 3$ (1a-10-3) and 5 (1a-21-3). See also Fig. S2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

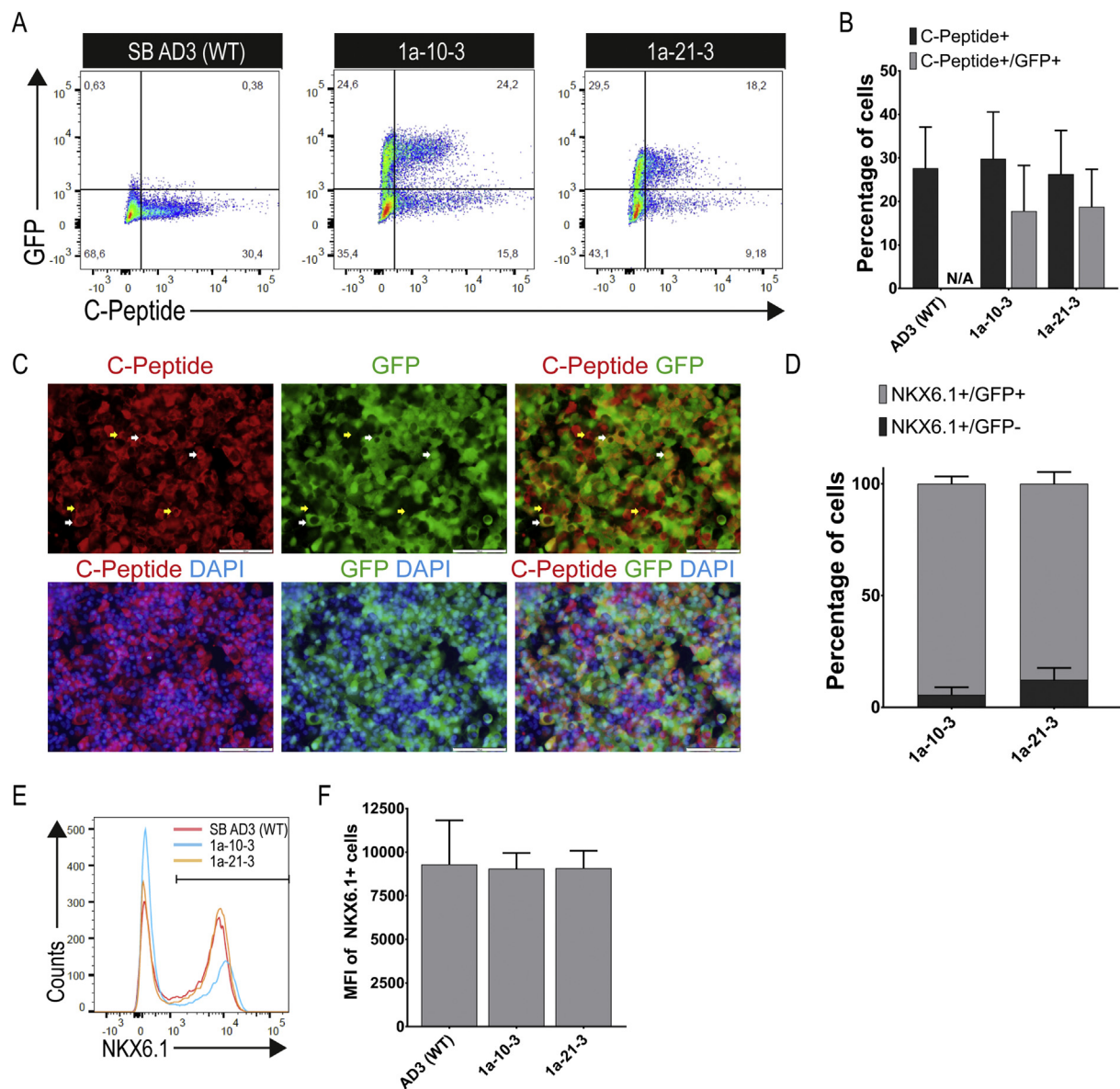


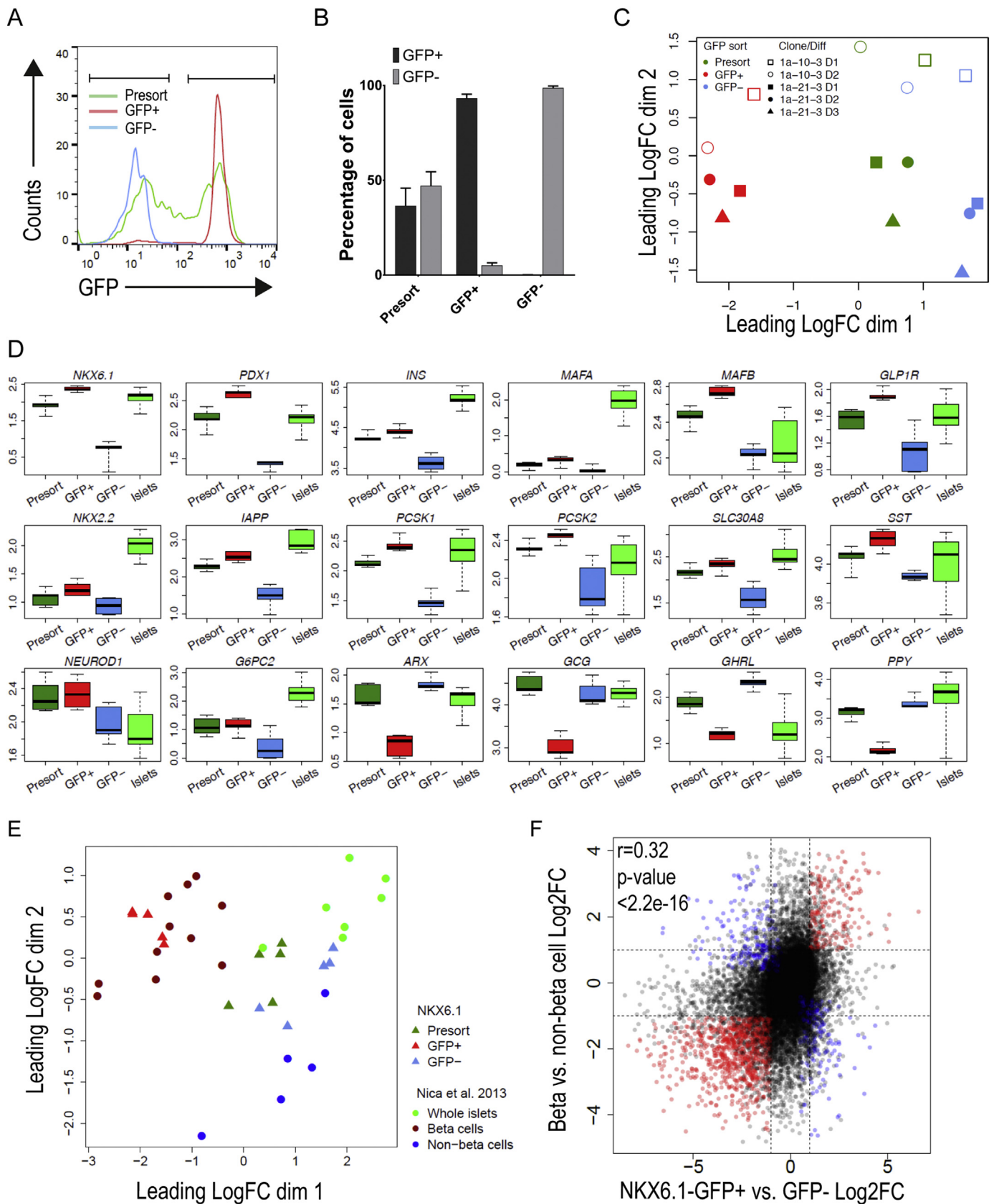
Fig. 3. Differentiation of NKX6.1-GFP lines to insulin-producing beta-like cells. A). Representative flow cytometry dot-plots showing expression of C-peptide and GFP in wild type (SB AD3) and NKX6.1-GFP iPSC lines (1a-10-3 and 1a-21-3) differentiated to stage 7 of the differentiation protocol. B) Quantification of C-peptide expressing and C-peptide/GFP co-expressing cells at stage 7. Bars shows mean \pm SD, 1a-10-3 (n = 3), 1a-21-3 (n = 4), AD3 WT (n = 5). C) Representative immunofluorescence images of 1a-10-3 at stage 7 of protocol stained with C-peptide and GFP antibodies. Nuclei revealed with DAPI. White arrows points to C-peptide+/GFP+ cells, yellow arrows show cells only expressing C-peptide. Scalebar = 100uM. Similar results obtained with 1a-21-3 (n = 2 for each line). D) Quantification of NKX6.1 and GFP cells by flow cytometry at stage 7. Bars shows mean \pm SD of number of NKX6.1+/GFP- (dark grey) and NKX6.1+/GFP+ (light grey), 1a-10-3 (n = 2), 1a-21-3 (n = 4). E) Representative histogram of NKX6.1 expression in wild type (red line) and NKX6.1-GFP reporter lines (blue and yellow line). Gate set to quantify number of NKX6.1 expressing cells based on negative control (not shown). F) Quantification of NKX6.1 expressing cells as shown in 4E. Bars shows mean \pm SD of the mean fluorescence intensity (MFI) of NKX6.1 signal in the NKX6.1 expressing cells, 1a-10-3 (n = 2), 1a-21-3 (n = 4), AD3 WT (n = 5). See also Fig. S2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

differentiation of both lines into motor neuron lineage. Importantly, we noticed an absolute overlap between ISL1-positive cells with Tuj1 staining indicating that the ISL1 expressing cells are legitimate neurons (Fig. 6B). In summary, these results prove that the reporter lines faithfully report NKX6.1 expression during differentiation towards the motor neuron lineage.

4. Discussion

Here we describe the generation and characterization of NKX6.1-GFP reporter iPSC lines with insertion of the *GFP* gene following the endogenous *NKX6.1* locus using CRISPR-Cas9 aided homologous

recombination. *NKX6.1* has previously been shown to play a critical role in the development and function of pancreatic beta cells (Taylor et al., 2013; Schaffer et al., 2013) as well as motor neurons (Sander et al., 2000). Our results demonstrate that the NKX6.1-GFP reporter lines faithfully labelled NKX6.1 expressing cells during differentiation towards both pancreas and motor neuron lineages, with > 80% of the NKX6.1-positive cells co-expressing GFP throughout differentiation to beta-like cells. An additional advantage of the reporter system presented here is that the NKX6.1-T2A-GFP construct is not expected to disrupt the endogenous *NKX6.1* loci but still allows for expression of GFP under the control of the *NKX6.1* promotor. We confirmed this by comparing the levels of NKX6.1 protein expressed in the wild type iPSC



(caption on next page)

Fig. 4. Transcriptome profiling of NKX6.1-positive cells isolated from a heterogeneous cell culture. A) Histogram showing GFP signal intensity in pre-sorted cells (red line), purified GFP-positive cells (blue line) and GFP-negative cells (orange line). Gates shown in histogram labels GFP + and GFP- cell populations respectively. Histogram representative of 5 experiments with similar results. B) Percentage of GFP + (dark grey bars) and GFP- (light grey bars) cells in the presorted, GFP + and GFP- populations as defined in Fig. 4A. Bar shows mean \pm SD, $n = 5$. C) Multidimensional scaling plot of the whole transcriptome RNA-seq data from multiple independent differentiations (marked with different symbols) of the two NKX6.1-GFP clones (1a-10-3 marked with empty and 1a-21-3 with filled-out symbols) of the NKX6.1-GFP cell line differentiated to beta-like cell stage (stage 7). D) Gene expression of differentiation and cell type markers in the RNA-seq data from sorted NKX6.1-GFP cell populations as well as previously published data set on human islets - reported in Log10 (TPMs). Individual biological replicates are shown as box and whiskers plots ($n = 5$). E) Multidimensional scaling comparison of the transcriptomic profiles of the sorted NKX6.1-GFP cell populations to previously published transcriptome profiles of whole pancreatic islets, as well as sorted beta and non-beta cells. F) Comparison of log2 fold changes of gene expression between NKX6.1-GFP + and GFP- cell populations and the previously published beta and non-beta cell populations. The log2 fold changes from the two experiments are significantly correlated ($r = 0.32$, p -value $< 2.2 \times 10^{-16}$). For genes with absolute fold changes > 2 in both studies, red marks genes with fold changes concordant in both experiments, whereas blue marks genes with opposite signs of fold change. See also Fig. S3 and S4. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

line with that of the two reporter lines and found no significant difference. Thus, these NKX6.1-GFP iPSC lines provide a useful tool for implementing and optimizing differentiation protocols, and delineating the role of *NKX6.1* in both motor neuron and pancreas progenitor cells, as well as in more mature beta-like cells.

Despite the recent advances in the generation of beta-like cells from hPSC, the cultures often contain a heterogeneous mix of cell populations, including beta-like cells, polyhormonal cells expressing several hormones and other undefined cell types. The reporter cell lines described here can be used to reduce the heterogeneity of the cell cultures

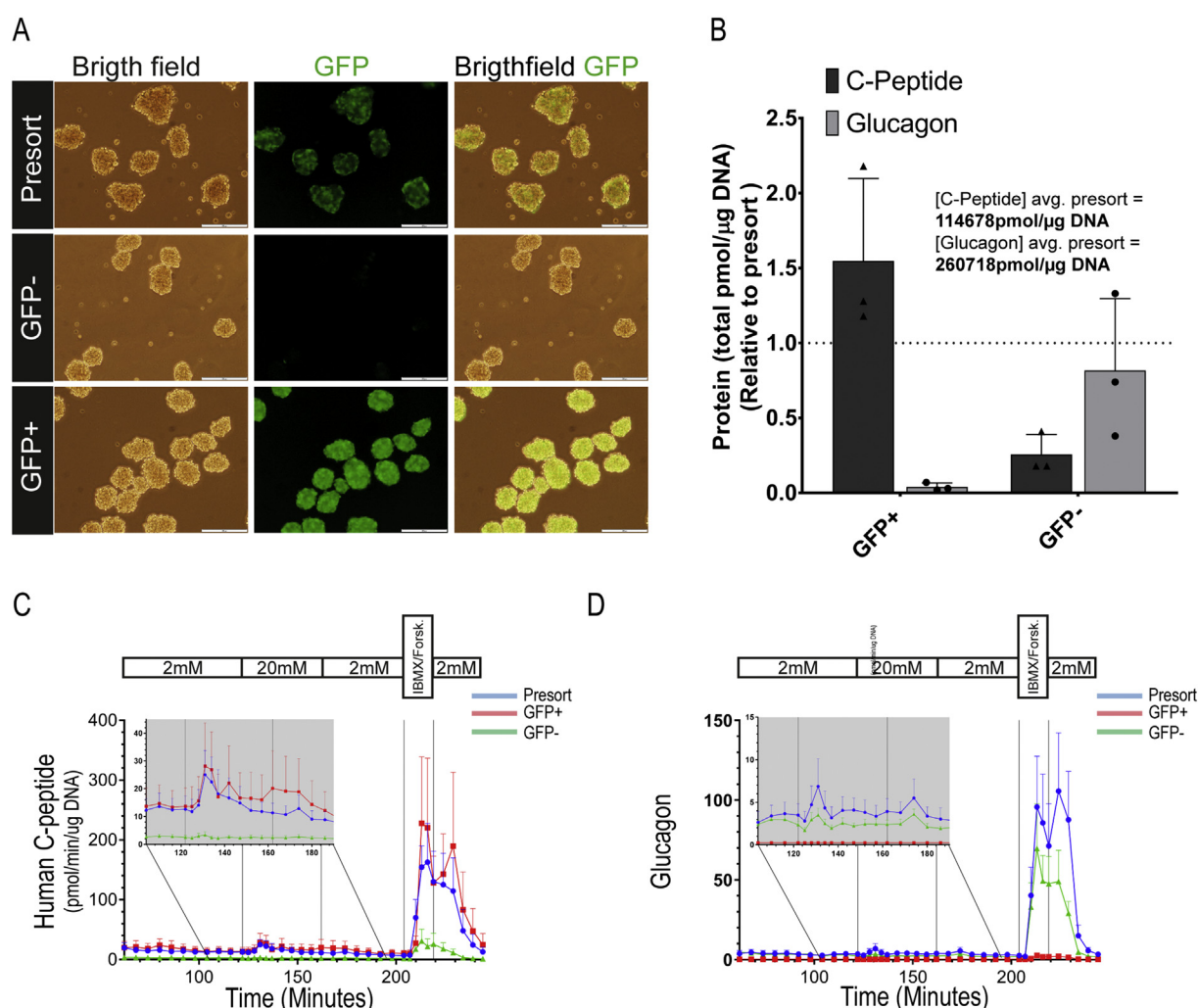


Fig. 5. Functional evaluation of purified cells reveals glucose-responsive cells within the NKX6.1-positive population. A) Bright field and GFP fluorescence microscopy of unsorted (presort) and FACS sorted GFP negative and positive populations following overnight aggregation in low attachment dishes. GFP signal in all populations was acquired using the same exposure time. Representative images of $n = 4$ biological experiments. B) Total C-peptide and glucagon content in GFP positive (GFP +) and GFP negative (GFP-) populations following perfusion experiments. Graph shows mean \pm SD of $n = 3$ with individual biological experiments shown as symbols. Values are normalized to the content of the pre-sorted population (dashed line). The values for total C-peptide and Glucagon in pre-sorted population are displayed. C-D) Measurements of secreted C-peptide (C) and Glucagon (D) from unsorted population (blue line), GFP positive (red line) and GFP negative (green line) shown as pmol pr. min pr. μg DNA of loaded cells. Graph shows mean \pm SEM of three perfusion experiments with cells from three individual differentiations in total of the two NKX6.1-GFP iPSC lines ($n = 3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

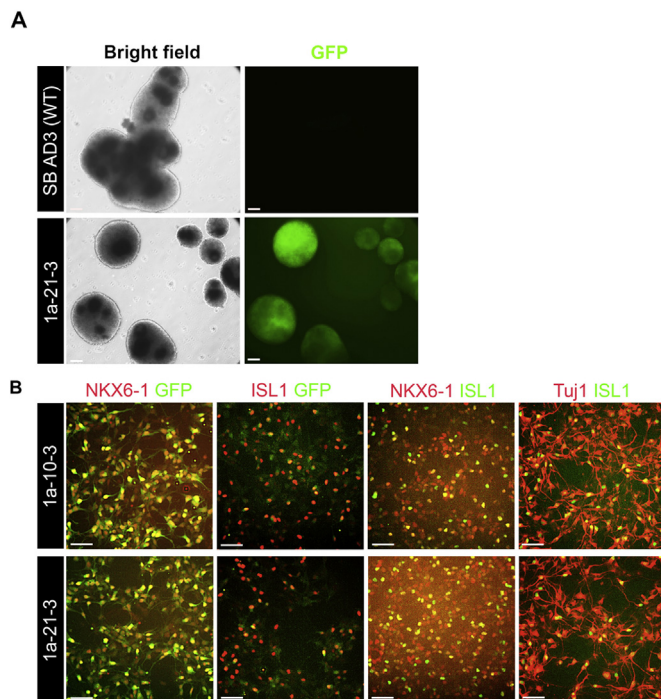


Fig. 6. Differentiation of NKX6.1-GFP line into Motor Neuron (MN) lineage. NKX6.1-GFP iPSC lines (1a-10-3 and 1a-21-3) were differentiated into motor neuron lineage and analysed for GFP expression and MN markers by immunofluorescence microscopy. A) Representative bright field and fluorescence microscopy images of live cultures of wild-type and 1a-21-3 Embryoid Bodies (EBs). Scale bar = 100 μ M. B) Differentiated cells from NKX6.1-GFP clones (1a-10-3 and 1a-21-3) stained for NKX6.1 & GFP protein, ISL1 & GFP protein, NKX6.1 and ISL1 and Tuj1 & ISL1. Scale bar = 100 μ M.

by enriching for *NKX6.1* expressing cells, thus allowing for the characterization of more pure cell populations. To demonstrate this, we isolated NKX6.1 positive and negative cells differentiated to pancreas endocrine cells to high purity and characterized their transcriptome profiles by RNA sequencing. Comparison with previously published data from isolated primary human islet cell populations revealed that the NKX6.1 positive cells derived from iPSC resembled the primary human beta cells more closely than the NKX6.1 negative and unsorted cell populations. This observation was further supported by the enrichment of several key beta cell gene transcripts such as *NKX6.1*, *PDX1*, *INS*, *GLP1R*, *IAPP*, *PCSK1* and *SLC30A8* in the NKX6.1 positive population.

We also examined the expression of genes specific for other endocrine cells and found that transcripts for the hormones *GCG*, *GHRL* and *PPY* were enriched in the NKX6.1 negative population. This suggest that cells resembling alpha, epsilon and PP cells are mainly located in this compartment, although it remains to be shown how similar these cell populations are to their human endocrine counterparts. To further support this, we noted that expression of the alpha cell determinant *ARX* was almost completely restricted to the NKX6.1 negative population, which is in agreement with a recent study examining the development of hPSC-derived pancreatic endocrine cells using single cell gene expression analysis (Petersen et al., 2017). NKX6.1 has previously been shown to negatively regulate expression of *ARX* and *GCG* in rodents (Schisler et al., 2005, 2013). Here we observed that both *ARX* and *GCG* transcripts, as well as glucagon protein were almost completely absent in the NKX6.1 expressing cells, which could suggest a similar antagonistic role in humans. Interestingly, expression of *SST* was observed both in the NKX6.1 positive and negative populations and we confirmed the expression of somatostatin in both GFP-positive and negative cells by both flow cytometry and immunofluorescence

imaging. Previous studies focusing on mouse pancreas development have suggested a bifurcation during endocrine development, with cells being directed towards either an alpha/PP or delta/beta progenitor cell before resolving into mature endocrine cells (Sosa-Pineda et al., 1997; Collombat et al., 2003; Kordowich et al., 2010). Our observations suggest that during development of human delta cells, these cells could arise from a progenitor cell expressing both somatostatin and NKX6.1. Supporting this notion, co-expression of NKX6.1 and somatostatin has previously been reported in foetal human pancreas (Gage et al., 2015).

While our results demonstrate that enriching for NKX6.1 positive cells in hPSC pancreatic differentiation cultures facilitates isolation of more pure populations of beta-like cells from heterogeneous cultures, the comparison with isolated primary human islet populations also revealed differences between the hPSC-derived beta-like cells and primary human beta cells. Notably, we observed that the level of *INS* transcript was significantly higher in human beta cells compared to any of the isolated populations from our differentiation cultures. Previous studies have shown that *INS* transcripts constitute between 30 and 50% of the total mRNA in beta cells (Segerstolpe et al., 2016; Nica et al., 2013), reflecting the high demand for insulin synthesis in these cells.

To date, the majority of the functional studies on hPSC-derived pancreatic endocrine cells have been performed on heterogeneous cell cultures containing both beta-like cells but also other endocrine cell types. Thus, it remains to be determined which cell population(s) are responsible for the functional readout assessed. The functional data performed on the isolated cells presented in this article represent two significant advances: firstly, we demonstrate that hPSC-derived beta-like cells can be isolated by FACS and subsequently maintained for functional analysis, and secondly, we demonstrate that cells capable of secreting insulin in response to elevated glucose concentrations are found primarily in the NKX6.1 positive population. Isolation of hESC-derived pancreatic lineages as well as primary human beta cell populations has previously been demonstrated using various sorting technologies (Kelly et al., 2011; Micallef et al., 2012; Dorrell et al., 2016). Building on the experience from these studies, we designed a strategy for FACS sorting and subsequent aggregation of the sorted, single cells. This allowed us to obtain highly pure NKX6.1-positive and negative cell populations that were amenable to functional evaluation following isolation. Interestingly, while we observed expression of insulin in all the NKX6.1 positive and negative, as well as the unsorted populations, secretion of insulin in response to glucose was restricted to the NKX6.1 positive and unsorted cells. Furthermore, we saw a consistent increase in secreted insulin from these cells when increasing the glucose concentration, suggesting that at least a portion of the NKX6.1 positive cells are able to secrete insulin in response to elevated glucose. In line with the gene expression profiling, these results further support that the beta-like cells generated in the *in vitro* differentiated cultures are restricted to the NKX6.1 positive compartment. Nonetheless, the amount of C-peptide/insulin secreted from the hPSC-derived beta-like cells was significantly lower compared to primary human islets. This could perhaps be a reflection of the lower amounts of *INS* expression observed in the hPSC-derived beta like cells compared to primary human islets. Thus, future strategies for generation of fully functional beta cells from hPSC could be directed at increasing the levels of *INS* expression as well as the amount of insulin secreted from these cells when exposed to increased glucose concentrations.

In summary, our results demonstrate that NKX6.1 positive beta-like cells can be purified using the NKX6.1-GFP iPSC reporter lines presented here in this study. We believe that these lines and the gene expression profiling reported here present a valuable resource for implementing and optimizing differentiation protocols towards both motor neuron and pancreatic lineages, as well as for uncovering novel aspects of human development. Furthermore, our strategy for performing functional experiments on FACS-purified populations could be applied to reporter lines expressing GFP or similar under the control of any gene of interest. This will hopefully allow for an even more

thorough understanding of the specific cell population(s) that contributes to the glucose responsive insulin secretion observed in the heterogeneous cell populations generated with the current and future differentiation protocols. The NKX6.1-GFP iPSC reporter lines presented here were developed and characterized as part of the IMI sponsored project StemBANCC. The lines will be distributed to the scientific community as a resource for motor neuron and pancreatic islets studies through the Coriell Institute for Medical Research iPS core bank and the European Bank for Induced Pluripotent Stem Cells (EBiSC).

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Declaration of interest

SKG, AKR, HJ, LS, CI, ATG, BM, MM, RRJ, JJF, LGG, MKT, RH, MH and CH are all employers in pharmaceutical companies (Eli Lilly, AstraZeneca, Novo Nordisk A/S) and may hold shares in these companies. ALG has received research funding from Novo Nordisk A/S. MIM is on advisory panels for Novo Nordisk A/S and Pfizer, Honoraria from Novo Nordisk A/S, Eli Lilly and Pfizer, and research funding from Novo Nordisk A/S, Eli Lilly, Pfizer, Merck, Janssen, Takeda, Roche, Boehringer Ingelheim, Sanofi Aventis and Servier.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scr.2018.04.010>.

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